Evaluation of the Performance of K-OTAB (Deltamethrin Tablet Formulation) Impregnated Bednets against the Malaria Vector *Anopheles* (*Cellia*) *superpictus* Grassi 1899 (Diptera: Culicidae) under Laboratory Conditions

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Abstract: The efficacy of K-OTAB (deltamethrin-tablet formulation) impregnated bednets was evaluated against the malaria vector *Anopheles (Cellia) superpictus* Grassi 1899 (Diptera: Culicidae) under laboratory conditions. The experiments were carried out with 3 main tests: susceptibility, bioassay, and washing tests. Deltamethrin treated nets in WHO test kits in susceptibility test 1 showed remarkable efficacy (89.5%) 1 h after the treatment and efficacy was 100% at the end of 24 h. The results demonstrated significant differences in mortality between different time intervals. Average mortality started to increase just after 15 min; it was 61% after 30 min, reaching 82% after 45 min and peaked to mass mortality rate at the end of 24 h. Efficacy against *An. superpictus* ranged from 90% to 100%, depending on different exposure periods in susceptibility test 2. The increase in mortality rates was exponential until 45 min exposure. The highest mortality rates were found at 45 and 60 min exposure times in the range of 95%-100%. Bioassay tests revealed gradual mortality ranged from 70% to 90%. The treatment efficacy was considered to be more dependent on the amount of washing than on the time after the treatment. The treated nets exhibited slightly poorer efficacy after the second and third washes, showing a decline from an initial value of 90% (unwashed) to 76.6% within 24 h of exposure after the second wash and to 75% after the third wash. The loss in effectiveness was within acceptable limits (13.4%-15%) compared with the loss in active ingredient (60%) after the second wash.

Key Words: Malaria, *Anopheles superpictus*, bednet, deltamethrin, laboratory tests

Laboratuvar Koşullarında K-OTAB (deltamethrin tablet formulasyonu) Emdirilmiş Cibinliklerin Sıtma Vektörü *Anopheles* (*Cellia*) *superpictus* Grassi 1899 (Diptera: Culicidae)'a Karşı Etkisinin Değerlendirilmesi

Özet: K-OTAB (deltametrin tablet formulasyonu) emdirilmiş cibinliklerin sıtma vektörü *Anopheles (Cellia) superpictus* Grassi 1899 (Diptera: Culicidae) üzerindeki etkinliği laboratuvar koşullarında incelenmiştir. Deneylerde üç temel yöntem (test) kullanılmıştır: hassasiyet, biyolojik etkinlik ve yıkama testi. WHO test kitlerinde deltametrin ile emdirilmiş cibinlikler, hassasiyet testi 1'de, uygulamadan 1 saat sonra çok yüksek düzeyde etki göstermiştir (% 89,5), 24 saat sonunda da ölüm oranı % 100'e ulaşmıştır. Sonuçlar, gerçekleşen ölüm oranlarının zaman aralıklarına göre önemli derecede farklı olduğunu ortaya koymuştur. Ortalama ölüm oranları, 15. dakikadan sonra artmaya başlamış, 30. dakikanın sonunda % 61'e, 45. dakikada % 82'ye yükselmiş ve 24 saat sonunda da en yüksek orana ulaşmıştır. Hassasiyet testi 2'de ise *An. superpictus* üzerindeki etkinlik oranları, maruz kalma sürelerine bağlı olarak % 90 ile % 100 arasında değişmiştir. Kırk beşinci dakikaya kadar ölüm oranlarındaki artış üssel bir biçimde ilerlemiştir. En yüksek ölüm oranları, 45. ve 60. dakikalarda bulunmuş ve % 95-100 arasında değişmiştir. Biyolojik etkinlik testlerinde ölüm oranları kademeli olarak gerçekleşmiş ve % 70-90 arasında gerçekleşmiştir. Etkinlik değerlerinin süreden çok yıkama ile ilişkili olduğu belirlenmiştir. İkinci ve 3. yıkamadan sonra deltametrin emdirilmiş cibinliklerde etkinlik kaybı gözlemlenmiş ve ikinci yıkamadan 24 saat sonra etkinlik değeri ilkin % 90'dan % 76,6'ya düşmüş, üçüncü yıkamadan sonrada bu değer % 75'e gerilemiştir. Aktif maddedeki azalma ile karşılaştırıldığında ikinci yıkamadan sonraki etkinlik değerinde kabul edilebilir bir düşüş olduğu belirlenmiştir (% 13,4-15).

Anahtar Sözcükler: Sıtma, *Anopheles superpictus*, cibinlik, deltametrin, laboratuvar testleri

Introduction

Anopheles superpictus is a widespread species in the Palaearctic Region (Knight and Stone, 1977) and is considered an important malaria vector throughout its distribution area (Barkai and Saliternik, 1968; Zahar, 1974; Romi et al., 1997). It is also one of the most widely distributed species vertically, occurring from sea level to over 1700 m in Turkey (Parrish, 1959; Postiglione et al., 1973; Alten and Çağlar, 1998; Özer et al., 2001). Because of its exophilic and zoophilic tendencies, the vectorial role varies according to the social and biological characteristic of the regions in Anatolia (Postiglione et al., 1973). On the other hand, the importance of *An. superpictus* as a vector of malaria has not been thoroughly studied. Although no naturally infected or infective female has been found in Turkey, the efficiency of this species as a vector of *Plasmodium vivax* (Kasap et al., 1987) and *Plasmodium falciparum* (Luty et al., 2006) has been demonstrated under laboratory conditions.

During the past decade, pyrethroid-treated nets have become established as an important defence against malaria transmission (Curtis et al., 1990; Choi et al., 1995; Lengeler et al., 1998). Large-scale epidemiological field trials involving community use of pyrethroid-impregnated bednets have demonstrated major benefits in reducing malaria morbidity and mortality (Snow et al., 1988; Nevill et al., 1996; Rashed et al., 2000; Armstrong-Schellenberg et al., 2001; Guyatt and Snow, 2002; Alten et al., 2003a). In addition, there are many studies on the efficacy, under laboratory conditions, of pyrethroid insecticides for impregnation of mosquito nets (Ansari et al., 1998; Kolaczinski and Curtis, 2000; Adams et al., 2002).

Although conventional malaria control programmes using residual insecticide spraying have been conducted by the Turkish Ministry of Health in collaboration with municipalities and private companies, apart from case findings and treatment studies there is little information on feasible means of controlling *Anopheles* species and malaria in Turkey. The only reported attempt was a trial of insecticide house-spraying and fogging of related areas. This brought a transient reduction but rapid re-invasion of the vector from unsprayed areas. However, where appropriate, this method of control is rapidly being complemented globally by the use of insecticide-impregnated bednets. Turkey has still little experience in the use of insecticide-impregnated bednets.

Deltamethrin has proved to be one of the most effective insecticides, especially under varying climatic conditions in Turkey (Alten and Çağlar, 1998), and is already the most widely used insecticide for impregnating bednets for effective long-term control of malaria (Curtis et al., 1990; Alexander et al., 1995; Hougard et al., 2003). The effect of the use of bednets treated with deltamethrin (K-OTAB, tablet formulation containing 25% deltamethrin) on the main malaria vector, *An. sacharovi*, has been also studied extensively at community levels in Şanlıurfa province, Turkey (Alten et al., 2003a). The results showed a significant reduction in malaria incidence in experimental areas from 8.29% in the pre-treatment year to 1.57% in the post-treatment year. The present study evaluates the effectiveness of bednets impregnated with tablet formulation (K-OTAB) against another proven malaria vector in Turkey, *An. superpictus*, under laboratory conditions.

Materials and Methods

The efficacy of a pyrethroid used for the impregnation of mosquito nets is the result of the insecticide's intrinsic activity and the behaviour of the target mosquito in response to it. This is of particular relevance for fast-acting insecticides with knock-down and irritant properties (Hougard et al., 2003). The intrinsic activity can be tested with adult mosquitoes using a device which forces tarsal contact with the impregnated netting material. This test does not indicate overall insecticide efficacy under field conditions, however, because the forced contact does not permit natural avoidance behaviour.

The experiments to evaluate the efficacy of tablet formulation of deltamethrin on mosquito nets against *An. superpictus* were carried out under laboratory conditions with 3 main procedures: bioassay tests, susceptibility tests and washing tests.

Mosquitoes

The mosquitoes used in the experiments were drawn from a laboratory colony of *An. superpictus* (F_{60} progeny) originating from Şanlıurfa, a malaria endemic province in Turkey near the border with Syria. The colony is robust and susceptible to deltamethrin and other insecticides used in Turkey. Blood-fed females aged 3-4 days from a single cohort were used in the experiments. The mosquitoes were kept in cages provided with 10%

sucrose solution at a temperature 27 ± 2 °C and 70 ± 5% RH with a 12:12 h (L:D) photoperiod regime. Dawn and dusk phases were supplemented with automatically dimmed fluorescent bulbs (40 W) activated twice: 06:00-07:00 and 18:00-19:00. On the morning of the day of an experiment, healthy females were aspirated from the stock cages and placed in paper cups with gauze lids. Each cup contained 30 females and was provided with a pad of cotton wool saturated with 10% sucrose solution. A sufficient number of cups were prepared and labelled for the day's trial and left in the insectarium until used in exposures.

Substrate and treatment

Tests were conducted using netting material [warp-knitted multifilament polyester 100 denier, mesh 156 (Siamdutch, Thailand)] treated with K-OTAB (1.6 g tablet containing 0.4 g deltamethrin active ingredient) at 25 mg a.i./m^2. A tablet was dissolved in 500 ml of deionised water. The solution was used for one bednet, which was family size, totally 11.64 m^2, at the rate of 25 mg/m^2. The bednets were dipped into 500 ml of the appropriate dilution of tablet formulation. A 50-l plastic bath was used for the dipping procedure and the final quantity of solution used for each treatment was arrived at by measuring the quantity of water taken up by a single net. The net was vigorously agitated in it by hand. The person performing this procedure wore plastic gloves. When no further solution was taken up by the net, it was removed to a well-ventilated room and allowed to drip dry and left to dry overnight in the dark. On the morning of the day of experiment, 10 pieces (12 cm x 15 cm) were cut from each bednet, the pieces of net were stapled to Whatman chromatography paper and placed into labelled WHO mosquito cylinders to use in the experiments. The same procedure was applied to prepare pieces of standard control using deionised water.

Washing procedure

After each exposure, one of the nets from each treatment was washed with soap powder for washing tests. Two scoops of soap powder were added to 2.5 l of hot water (40 ± 2 °C) in a glass container. Bednets treated with the same formulation were added and stirred twice. After 5 min of soaking, the nets were stirred a further 6 times and then soaked for another 5 min. The nets were then squeezed 6 times and, after a total of 12 min in the washing water, the nets were

removed and rinsed twice in cool water. The bednets were allowed to dry for at least 24 h prior to the next wash. The treated nets were unwashed, washed once, washed twice or washed 3 times prior to testing. On the morning of each experimental day, the same procedure mentioned above was used before the pieces were placed into WHO cylinders.

Susceptibility tests 1-2

Batches of 20 healthy females from resting cups were supplied in green spot WHO cylinders lined with insecticide-free paper. These were attached to red spot cylinders containing the treated bednet pieces. The insects were gently blown from the green spot cylinder into the treated bednet side and exposed to the bednet for 60 min. During the exposure period, mortality was recorded at 3, 15, 30, 45 and 60 min. When the exposure time was complete, the insects were transferred back into labelled paper cups and held for 24 h at 27 ± 2 °C and 70 ± 5% RH with 5% sugar solution provided. Mortality was assessed 24 h post-exposure to the treated bednets. Deltamethrin susceptibility of *An. superpictus* was tested using a total of 240 females consisting of 10 replicates of 20 females each to allow for inter-batch variation with 2 replicates for standard control.

The same procedure described above for test 1 was used in test 2 with the following differences: exposures were grouped into 5 different time periods: 3, 15, 30, 45 and 60 min. After each exposure, the insects were gently transferred into labelled paper with 5% sugar solution provided. Mortality was recorded 1 and 24 h post-exposure to the treated and control bednets. Each time period was tested using a total of 120 mosquitoes consisting of 5 replicates of 20 females with a replicate for standard control and total of 600 females were used during test 2.

Bioassay test

Knock-down effect and mortality resulting from tarsal contact with netting material were measured using the same kits and procedures in susceptibility tests mentioned above with the following differences: mosquitoes were exposed for 3 min to the treated netting and to standard controls. They were then removed and left in clean paper glasses with gauze lids. Knock-down was then assessed at 3, 5, 10, 15, 30, 45 and 60 min and mortality was recorded 24 h post-exposure to the treated and control bednets. Washed and unwashed nets were used for

bioassay tests against *An. superpictus.* Each condition was tested using a total of 80 mosquitoes consisting of 3 replicates of 20 females with a replicate for standard control and total of 320 females were used during the bioassay tests.

Chemical analysis

A total of 72 unwashed and washed pieces consisting of 3 replicates in each side were cut from different sides (top, bottom, right side, left side, front and behind) of impregnated bednets. The netting was removed, folded into aluminium foil sachets, labelled and stored in sealed plastic bags at 4 °C for chemical analysis. They were analysed by Normal Phase HPLC (after extraction with 90/10 Isooctane/THF) on a Chromospher silica column. The mobile phase was 97/3 Isooctane/1.4 Dioxan and UV detection was at 236 nm.

Statistical analysis

The significance of possible sources of error was assessed by Kruskal-Wallis one-way analysis of variance (ANOVA) according to Zar (1996). The effect of insecticide was considered significantly different when the P value was less than 0.05. The tests were conducted in parallel with a control with no insecticide. Mortality rates observed after 24 h were corrected using the Abbott formula.

Results

At the termination of the experiments, the average mortality rates and their standard deviations were calculated for susceptibility tests 1 and 2 and the results are shown in Figure 1 and 2 separately for the control and test groups. As expected, the Kruskal-Wallis test showed that there were highly significant differences in mortality rates between the test group treated with the K-OTAB and the untreated control for all comparisons (H = 31.73 at P < 0.001). In addition, although some small differences due to individual variations in the mosquito cohorts used in the experiments were diagnosed, there were no significant differences among the replicates (H = 0.53 at P > 0.05). Tablet formulation of Deltamethrin (K-OTAB) in susceptibility test 1 showed remarkable efficacy (89.5%) 1 h after the treatment and efficacy reached 100% at the end of 24 h. When the results were scrutinised, there was a very low mortality rate (5%) during the first 3 min; however, during the first 15 min a small proportion (12%) of the mosquitoes died. In this

experiment, there was a statistically significant difference between different time intervals of the study period in terms of mortality rate in the treated groups (H = 27.67 at P < 0.001). However, average mortality started to increase just after 15 min; it was 61% after 30 min, reaching 82% at 45 min, and peaked at the mass mortality rate at the end of 24 h (Figure 1).

Figure 2 indicates that there was no significant difference between different exposure times in terms of mortality rate in the treated groups in susceptibility test
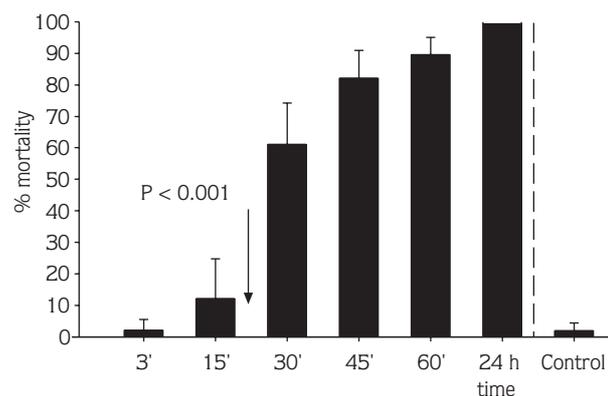

Figure 1. Lethal effect of deltamethrin tablet formulation (K-OTAB) at the rate of 25 mg ai/m^2 after 60-min exposure against the malaria vector *Anopheles superpictus* under laboratory conditions.
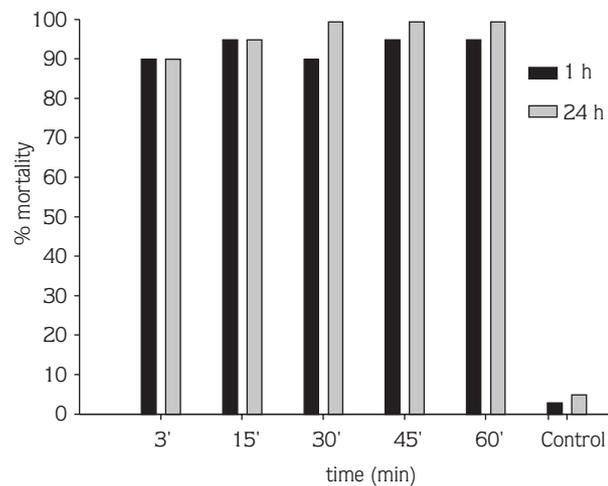

Figure 2. Mortality of *Anopheles superpictus* after exposures grouped into 5 different time periods to tablet formulation of deltamethrin (K-OTAB, 25 mg ai/m^2) on polyethylene netting under laboratory conditions.

2 (H = 1.21 at P > 0.05). In contrast, a significant difference was found between the test group treated with the K-OTAB and the untreated control for all comparisons (H = 15.44 at P < 0.001). The efficacy of the K-OTAB against *An. superpictus* ranged from 90% to 100%, depending on different exposure periods. The mosquitoes exposed to 3 min contact with the pieces of treated bednets showed 90% mortality at the end of both 60 min and 24 h. The increase in mortality rates according to exposure time was also exponential until 45 min exposure. The highest mortality rates were found at 45 and 60 min, in the range 95%-100%.

Bioassay tests revealed gradual mortality ranging from 70% to 90% according to different time intervals and washing. Figure 3 shows the mortalities in bioassays with *An. superpictus* of the washed and unwashed nets whose deltamethrin content is shown in the Table. Although the loss of compound tested after 3 washes was around 60%, no significant difference was found in the mortality rate between the unwashed and washed test groups (H= 1.20 at P > 0.05). The unwashed nets caused 50% mortality within 30 min of exposure and 63% and 90% mortality rates after 60 min and 24 h, respectively. The increase in mortality rates was also found to be exponential for each condition depending on time. In contrast, the treated nets exhibited slightly poorer efficacy after the second and third washes, showing a decline from an initial value of 90% (unwashed) to 76.6% within 24 h of exposure after the second wash and to 75% after the third wash (Figure 3). This indicates that with this very powerful pyrethroid even the low initial doses applied had a considerable effect and one could afford to wash out a large fraction of the active ingredient without losing the ability to kill 75% of mosquitoes with 3 min exposure.

Discussion

The spraying of residual insecticides on the inner walls of houses and barns has been used to control malaria vectors as the main method organised by central authorities for several decades in rural and urban areas in Turkey. Although residual spraying of walls with insecticides such as deltamethrin has also been shown to be effective against malaria vectors (WHO, 1993), certain vector species continued to bite volunteers inside houses with sprayed walls, probably before alighting on the treated surface (Alten and Çağlar, 2001). It is thought that anti-malarial insecticide spraying as a part of
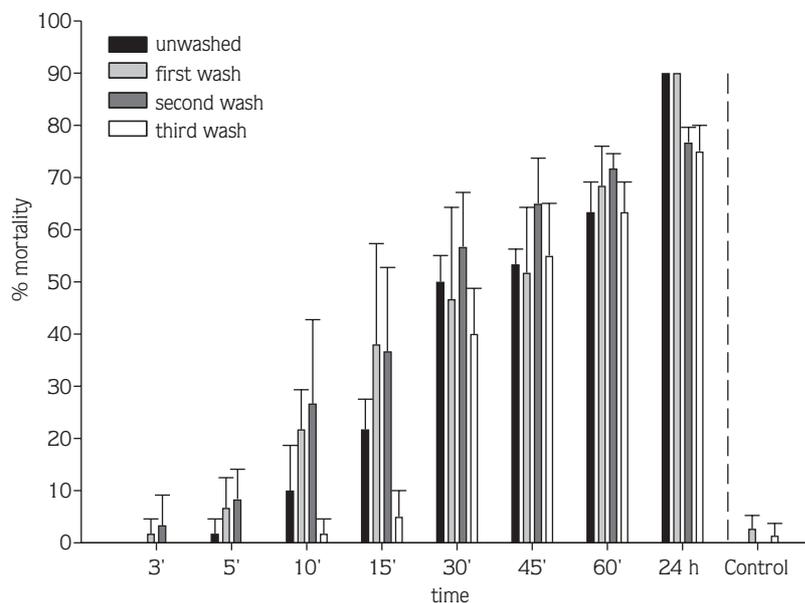

Figure 3. Mortality of *Anopheles superpictus* after 3-min exposure to tablet formulation of deltamethrin (K-OTAB, 25 mg ai/m^2) on polyethylene netting which was either unwashed or washed up to 3 times under laboratory conditions.

Table. The results of HPLC analysis of the bednet used in the experiments.

| position of pieces | dosage (mg/m^2) | | | |
| | number of washes | | | |
	unwashed	1	2	3
top	25.95*	21.21	15.15	10.17
bottom	25.42	18.99	13.17	9.77
right side	27.51	17.21	14.22	8.75
left side	23.13	19.19	13.28	11.23
front	24.44	20.17	13.99	9.11
behind	26.15	18.33	14.78	7.99

* average value of bednet pieces

integrated vector control programmes has still contributed to a considerable decline in malaria incidence, and the insecticides used in these programmes play an important role in controlling malaria vectors in many countries. On the other hand, residual insecticide application in itself does not produce the desired effects in malaria control in Turkey (Alten et al., 2003a) because of many reasons, such as lack of trained manpower, inadequate logistics support, corruption, mismanagement of the extensive insecticide product, and resistance of some vector populations to certain insecticides. In addition, the existence of exophilic vector species in especially malaria endemic areas has been also an important setback.

An. sacharovi, the principal human malaria vector in Turkey, and *An. superpictus* exploit different larval habitats and thus have different geographical distributions. Kasap et al. (1987) demonstrated for the first time experimentally that *P. vivax* from south-eastern Anatolia, Turkey, developed and completed sporogony in *An. superpictus*, establishing this species as an efficient laboratory vector. In laboratory conditions, *P. falciparum* from laboratory culture has been also demonstrated in this species (Luty et al., 2006). However, adults of both species may coexist temporarily, although *An. superpictus* is more exophilic (Postiglione et al., 1973). Due to its exophilism, the conventional residual spraying is insufficient to kill populations of this species. Ramsdale and Hass (1978), Alten et al. (2003b) and Şimşek et al. (2005) concluded that people sleeping outside of villages

and towns were more vulnerable to bites by *An. superpictus* and, in such situations in south-western Turkey, this species was able to maintain malaria transmission. Furthermore, we know that *An. superpictus* would bite humans more frequently in places where animals are not abundant, thus becoming an important malaria vector.

Our previous studies at community level on the main malaria vector, *An. sacharovi*, showed that deltamethrin impregnated bednets (K-OTAB, tablet formulation containing 25% deltamethrin) produced major effects in reducing malaria incidence in experimental areas from 8.29% in the pre-treatment year to 1.57% in the post-treatment year in a malaria endemic area (Şanlıurfa province) in Turkey (Alten et al., 2003a). After *An. superpictus* was shown to be transmitting *P. falciparum* and its vectorial capacity was demonstrated extensively, our attention has been focused on the practice of using impregnated bednets as one of the best personal protection methods for the control of *An. superpictus* transmitting malaria in open and hilly areas where the species exist abundantly.

As discussed in similar studies, the effectiveness of the treated nets can be reduced by using an inadequate dosage, and from loss or reduced dosage of insecticide due to the washing of treated nets. The treatment efficacy is considered more dependent on the amount of washing than on the time after the treatment (Curtis et al., 1990; WHO, 1997). Generally, nets that are freshly treated with the recommended dose of an insecticide are

expected to yield almost 100% mortality of target anophelines. After a period of use, and in particular after washing, the mortality tends to decline (WHO, 1997; Lengeler et al., 1998; Armstrong-Schellenberg et al., 2001, Rassi et al., 2002; Alten et al., 2003a, 2003b). Even if hand washing in cold and soapy water was the best method for cleaning the treated nets (Snow et al., 1987), chemical analysis showed that about half the initial content of a variety of deltamethrin was lost in this experiment. However, the results of bioassay obtained demonstrated that even after washing the bednets, the bioefficacy was still relatively high in mortality. The loss in effectiveness of tablet formulation against *An. superpictus* was within acceptable limits (13.4-15%) compared with the loss in active ingredient (60%) after the second wash. Hougard et al. (2003) showed that mosquito nets were still effective after 3 to 4 washes.

In testing for the lethal effect of insecticides it is desirable to use a short exposure period so that knock-down does not begin until exposure is completed. This avoids some mosquitoes spending part of the intended exposure period lying on their backs on or off the impregnated netting, thus absorbing either more or less insecticide than if they spent the whole period absorbing it through their tarsi (Schreck et al., 1978). However, we found in susceptibility test 1 that average mortality reached 82% from 61% after 30 min. The increase in mortality rates according to exposure time was also

exponential until 45 min exposure in susceptibility test 2. The instability due to the relatively low mortality rate determined in the first 15 min also appeared in the standard deviation value. This was related to the high variation between the mortality rates and the seldom deaths in the mentioned period of time. For this reason, we may say that as the active ingredients increased the mosquitoes' tendency to avoid contact with the repellency no knock-down effect was seen at the beginning. The authors' experience is that an exposure of 30 min would be reasonable for laboratory determination. In fact, the value of KDT_{50} (median knock-down time) for K-OTAB was in the range 22-27 min.

According to the results of these laboratory experiments, it could be concluded that in particular areas with the presence of different vectors with exophilic and exophagic habits, such as *An. superpictus*, and a long transmission season, the use of impregnated bednets with deltamethrin tablet formulation could reduce the incidence of malaria. Our previous studies clearly demonstrated that the assessment of user acceptability of the tablet formulation has shown a good acceptance by the population in urban parts of a malaria endemic region (Alten et al., 2003a). Further studies should quantify the impact of this personal control method on *An. superpictus* population density and assess the feasibility of method at community level in the open and hilly rural areas of south-eastern Anatolia.

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